

Notch3 Inhibits Epithelial-to-Mesenchymal Transition by Transactivating Glycogen Synthase Kinase-3-beta–Mediated Wnt/ β -catenin Signaling in Breast Cancer

Wei-Ling Chen

Xiamen University

Yong-Qu Zhang

Xiamen University

Rong-Hui Li

Xiamen University

Wen-He Huang

Xiamen University

Xiao-Long Wei

Shantou University Medical College

De Zeng

Shantou University Medical College

Yuan-Ke Liang

Shantou University Medical College

Yun-Zhu Zeng

Shantou University Medical College

Yao-Chen Li

Shantou University Medical College

Guo-Jun Zhang (✉ guoj_zhang@xah.xmu.edu.cn)

Xiang'an Hospital of Xiamen University <https://orcid.org/0000-0001-5182-5887>

Research Article

Keywords: breast cancer, Notch3, GSK3 β , epithelial-to-mesenchymal transition, prognosis

Posted Date: January 18th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1251054/v1>

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Abstract

As a critical transformational process in the attributes of epithelial cells, epithelial-to-mesenchymal transition (EMT) is involved in tumor invasion, metastasis, and resistance to treatment, which contributes to the ultimate death of some patients with breast cancer. Glycogen synthase kinase-3-beta (GSK3 β) is thought to be an EMT suppressor that down-regulates the protein, snail, a zinc finger transcription inhibitor, and regulates E-cadherin expression and the Wnt signaling pathway. Our previous studies have shown that Notch3 also inhibits EMT in breast cancer. In mammary gland cells, GSK3 β physically bound and phosphorylated the intracellular domain of two Notch paralogs: N1ICD was positively regulated but N2ICD was negatively regulated. However, the relationship between Notch3, GSK3 β , and EMT in breast cancer is still unclear and crosstalk between Notch3 and GSK3 β has not been widely investigated.

In this study, we revealed that Notch3 was an essential antagonist of EMT in breast cancer cells by transcriptionally upregulating GSK3 β . In breast cancer MCF-7 and MDA-MB-231 cell lines, the silencing of Notch3 reduced GSK3 β expression, which is sufficient to induce EMT. Conversely, ectopic Notch3 expression re-activated GSK3 β and E-cadherin. Mechanistically, Notch3 can bind to the GSK3 β promoter directly and activate GSK3 β transcription. In human breast cancer samples, Notch3 expression is positively associated with GSK3 β ($r=0.416$, $P=0.001$). Moreover, high expressions of Notch3 and GSK3 β mRNA are correlated to better relapse-free survival in all breast cancer patients via analysis in “the Kaplan–Meier plotter” database.

In summary, our preliminary results suggested that Notch3 might inhibit EMT by trans-activating GSK3 β in breast cancer cells. The suppression of Notch3 expression may contribute to EMT by transcriptionally downregulating GSK3 β in breast cancer.

Introduction

Breast cancer is the most common malignant tumor and main cause of death due to cancer worldwide in women. Moreover, local and/or distant metastasis is the leading cause of death in patients with this disease [1]. In recent decades, advances in early diagnosis, improved surgical techniques, and better adjuvant drug therapy have decreased the mortality rate of breast cancer [2]. Unfortunately, many patients exist who still have relapses or distant metastases, resulting in eventual treatment failure. The process of epithelial-to-mesenchymal transition (EMT) results in the transformation of epithelial cells into a mesenchymal phenotype, significantly reduces adhesion strength between cells, and drives the aggressiveness and cell migration of epithelial tumors [3]. In recent years, EMT has been associated with tumor genesis and progression, and is considered to be an important biological process leading to epithelial malignancy during embryonic development, with enhanced migration and invasion capacity [4]. The mechanisms involved in the occurrence and progression of EMT have been extensively explored, but other potential mechanisms, especially critical genes that drive EMT, still remain unclear [5]. As a highly conserved signaling pathway, Notch plays an essential role in numerous biological processes in metazoan development, including stem cell self-renewal, cell differentiation, proliferation, migration,

adherence, survival, apoptosis, and cell-fate decisions [6, 7]. Recently, increasing evidence has shown that Notch plays a fundamental role in development and tumorigenesis of mammary gland, including induction of the EMT process [8, 9]. Notch3, a member of the Notch family of transmembrane receptors, is considered an oncogene or tumor suppressor in distinct cancers [10-13]. Our previous studies showed that estrogen receptor (ER) α inhibits EMT by suppressing Bmi1 [14], and Notch3 transcriptionally upregulates ER α in breast cancer. Furthermore, Notch3 can inhibit EMT in breast cancer epithelial cells by transactivating Kibra [15]. The aforementioned results indicate that Notch3 inhibits progression of EMT in breast cancer.

Glycogen synthase kinase-3-beta (GSK3 β) is a serine/threonine kinase that can phosphorylate vast numbers of substrates, such as structural proteins, transcription factors, and signaling proteins [16]. Growing evidence demonstrates that GSK3 β acts as an EMT suppressor via its downregulation of snail, a zinc-finger transcriptional repressor that regulates the epithelial marker, E-cadherin [17]. Both Notch and Wnt pathways are known to play key roles in development and disease [6]. Direct or indirect interactions have been shown between these two signaling pathways [18, 19]. However, the relationship between Notch3 and GSK3 β in EMT of breast cancer is still unclear.

The purpose of this study was to investigate whether Notch3 acts as a transcriptional activator of GSK3 β in breast cancer. Notch3 was found to inhibit EMT by directly binding to the GSK3 β promoter and transactivating GSK3 β in breast cancer. In addition, Notch3 and GSK3 β expression was positively associated in human breast cancer samples. Finally, over-expression of both Notch3 and GSK3 β mRNA was found to be associated with better recurrence-free survival (RFS) in all patients with breast cancer.

Materials And Methods

Cell lines, plasmids, and reagents

Human breast cancer cell lines T-47D, MCF-7, MDA-MB-231, and BT-549, were purchased from the American Type Culture Collection (Manassas, VI, USA). The pCMV and pCMV-Notch3 intracellular domain (N3ICD) plasmids were given by Professor Michael M. Wang from the Medical School of Michigan University. The psi-U6.1/eGFP/short hairpin (sh)RNA-GSK3 β plasmid was purchased from GeneCopoeia (Guangzhou, China), and pCMV3-GSK3 β -GFPSpark was purchased from Sino Biological Inc (Beijing, China). A wild-type reporter assay vector containing a GSK3 β promoter region from -353 to -268 (p GL3-GSK3 β -enhancer) was generated and a mutant reporter assay vector was constructed by replacing a TCC (-307 to -305) sequence in the wild-type reporter assay vector with a DNA-binding protein (CSL) antisense binding sequence, TTCCCA. All antibodies used in this study are summarized in **Table S1**.

Plasmid DNA and siRNA transfection

We transfected 5 μ g of pCMV or pCMV-N3ICD into MDA-MB-231 cells using Lipofectamine 2000 or Lipofectamine 3000 (Invitrogen, Waltham, MA, USA). Stable Notch3-overexpressing clones were established using DMEM plus 10% fetal bovine serum (FBS) containing 1 mg/mL (selected

concentration) and 0.5 mg/mL (established concentration) of G418 (Merck&Co, Whitehouse Station, NJ, USA). In a rescue experiment, 2.5 µg of psi-U6.1/eGFP/shRNA-GSK3β was transfected into stable Notch3-overexpressing MDA-MB-231 cells growing in a 6-well cell culture plate for 48 h using Lipofectamine 3000 (Invitrogen). Control small interfering (si)RNA and Notch3 small interfering (si)RNA were synthesized by Gene Pharma Biotech (Shanghai, China). The siRNA sequences are listed in **Table S2**. Control or Notch3 siRNA (100 pmol) was transfected into 50–60% confluent MCF-7 cells. In a MCF-7 rescue experiment, 2 µg of pCMV3-GSK3β-GFPspark and 80 pmol of Notch3 siRNA were co-transfected into MCF-7 cells cultured for 48 h.

Western blot

Western blotting was performed as previously described [20]. The antibodies used are outlined in **Table S1**.

RNA extraction and RT-PCR analysis

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A PrimeScript RT Reagent Kit (Takara, Dalian, China) was used to synthesize first-strand cDNA. Gene expression was detected by quantitative real-time PCR analysis using a SYBR Select Master Mix (Applied Biosystems, Foster City, CA, USA). Sequences of all primers used in the study are summarized in **Table S3**.

Immunofluorescence

Immunofluorescence staining of MCF-7 and T-47D cells was performed as previously described [21]. Staining was visualized with a Zeiss microscope (Zeiss, Oberkochen, Germany). Notch3 and GSK3β antibodies are listed in Supplementary **Table S1**.

Wound healing assay

Wound healing assays were performed to evaluate the migration of MDA-MB-231 and MCF-7 cells under different transfection conditions. MDA-MB-231 cells were transfected with pCMV, pCMV-N3ICD or pCMV-N3ICD plus psi-U6.1/eGFP/shRNA-GSK3β, respectively, and inoculated in a 6-well cell culture plate at a density of 1×10^5 cells/well. Subsequently, cells were incubated in serum-free DMEM for 12 h. A pipette tip was used to establish a line in a monolayer of adherent cells. Photos were taken at 0, 24 h (MDA-MB-231) or 48 h (MCF-7). Similarly, MCF-7 cells were transfected with siRNA-negative control (NC), siRNA-Notch3, and siRNA-Notch3 plus pCMV3-GSK3β-GFPspark. Experiments were performed in triplicate with three follow-up fields recorded for each well.

Migration and invasion assays

BD Falcon Cell Culture Inserts (24-well plates, 8 µm; BD Biocoat, BD Biosciences, Franklin Lakes, NJ, USA) were used for cell migration assays as described previously [22, 23]. In an invasion assay, cells were

inoculated in the upper compartment of Matrigel-coated inserts (24-well plates, 8 μ m; BD Biocoat, BD Biosciences). All experiments were conducted in three separate assays.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as previously described [24, 25].

Sequences of all primers used in this study are shown in **Table S3**.

Construction of reporter assay vector and CSL-binding site mutation

A reporter assay vector, pGL3-GSK3 β -enhancer, containing a CSL-binding site region of the GSK3 β promoter was constructed to further explore the relationship between Notch3 and GSK3 β by dual-luciferase reporter assay. A pGL3-GSK3 β -enhancer-promoter mutant construct was generated by deleting three bases, "TCC", of the CSL-binding site in the wild-type pGL3-GSK3 β -enhancer-promoter vector using a site-directed mutagenesis kit (Cat. SDM-15, Saibaisheng Gene, China) according to the manufacturer's instructions.

Dual-luciferase reporter assays

To evaluate activity of the GSK3 β promoter activated by Notch3, we performed a dual-luciferase reporter assay. MDA-MB-231 and MCF-7 cells were inoculated into 24-well cell culture plates, and MDA-MB-231 cells were transiently transfected with pCMV, pCMV-N3ICD, pGL3-GSK3 β -enhancer-promoter reporter, and *Renilla* luciferase reporter plasmids using Lipofectamine 2000. Similarly, MCF-7 cells were transfected with siRNA-NC, siRNA-Notch3, pGL3-GSK3 β -enhancer-promoter reporter, and *Renilla* luciferase reporter plasmids using Lipofectamine 2000. Cells were harvested 48 h after transfection and measured as previously described [15].

Immunohistochemistry

Human breast cancer specimens were obtained from 68 patients who underwent breast cancer surgery at the Cancer Hospital of Shantou University Medical College in 2014. Immunohistochemical methods were undertaken as previously described [26].

The expression of Notch3 and GSK3 β was detected, with anti-mouse or rabbit IgG antibody (Abcam) used as negative controls. The immunohistochemical analysis criteria of Notch3 and GSK3 β were previously described [27]. Both Notch3 and GSK3 β stains localized in the cell nucleus and cytoplasm. The mean percentages of positive cells were scored as 0 (\leq 5%), 1 (5–24%), 2 (25–49%), 3 (50–74%), or 4 ($>$ 75%). The staining intensity was scored as 1 (weak), 2 (moderate), or 3 (strong). Final histological (h) scores were obtained for each case by multiplying the percentage and intensity score. Protein expression levels were further analyzed by classifying h values as low (based on an h value $<$ 5) or high (based on an h value \geq 5). The antibodies used are listed in Supplementary Table S1.

Database analysis

The patients' overall survival, relapse-free survival, and distal metastasis survival rate was based on Notch3 and GSK3 β expression levels, and automatically drawn by the database website's own software for Kaplan-Meier plots from the Kaplan–Meier Plotter (<http://kmplot.com>). The entire analysis process was performed in strict accordance of the site's instructions. The Breast Cancer Gene-Expression Miner v4.7 database (<http://bcgenex.centregauducheau.fr/BC-GEM/GEM-requete.php>) was used to explore the relationship, at the DNA level, between Notch 3 and GSK3 β .

Statistical analysis

Statistical analysis involved the use of SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Differences between variables were assessed by χ^2 analysis or two-tailed Student's *t*-test. Data are presented as the mean \pm standard error of the mean (SEM) unless otherwise indicated. Two-sided $P < 0.05$ was considered statistically significant. Each experiment was done at least in triplicate.

Results

Elevated expression of Notch 3 and GSK3 β correlated with a luminal subtype in breast cancer cell lines

We first performed an extensive analysis of the Breast Cancer Gene-Expression Miner v4.7 database in order to explore the relationship between Notch 3 and GSK3 β . We found that Notch3 was highly correlated with GSK3 β at the DNA level ($r = 0.15$, $P < 0.0001$; Fig. 1A, B). To further investigate the association of Notch3 and GSK3 β in breast cancer, we evaluated their protein expression levels in different subtypes of breast cancer cell lines. Notch3 protein was mainly present in the luminal subtype cell lines (T-47D and MCF-7) and almost absent in the triple-negative breast cancer (TNBC) cell lines (MDA-MB-231 and BT-549). Interestingly, GSK3 β expression paralleled Notch3 expression, with high levels observed in MCF-7 cells and nearly absent levels in MDA-MB-231 cells (Fig. 1C).

Ectopic Notch3 induces GSK3 β expression and inhibits EMT in human breast cancer cells

Increasing evidence shows that GSK3 β is an EMT suppressor and has been found to regulate Notch1 and Notch2 expression. We investigated whether cross-talk existed between Notch3 and GSK3 β in the progression of EMT in breast cancer. We found that the protein level of GSK3 β increased in ectopically overexpressed-Notch3 MDA-MB-231 cells but decreased in Notch3-knockdown MCF-7 cells (Fig. 2B, E). Similarly, quantitative real-time PCR showed that the GSK3 β mRNA level was consistent with the protein level detected by western blot (Fig. 2A, D).

To explore the role of Notch3 in breast cancer and its effect on EMT progression in breast cancer cells, we silenced endogenous Notch3 in MCF-7 cells using siRNA. Suppression of activated Notch3 (N3ICD) with siRNA significantly downregulated E-cadherin and upregulated vimentin, shown in the figure (Fig. 2C).

Conversely, Notch3 was ectopically overexpressed in MDA-MB-231 cells by stably co-transfecting PCLE/N3ICD; this revealed that Notch3 overexpression up-regulated E-cadherin but down-regulated vimentin (Fig. 2F). This noteworthy finding demonstrated that ectopic overexpression of Notch3 increased β -catenin phosphorylation (Thr41/Ser37/Ser33), and endogenous silencing of Notch3 decreased β -catenin phosphorylation (Thr41/Ser37/Ser33) (Fig. 2C, F). To further explore Notch3 and GSK3 β expression, we performed immunofluorescence staining for Notch3 and GSK3 β in MCF-7 and T-47D cells. As shown in Fig. 2G, Notch3 and GSK3 β co-localized in the luminal breast cancer cell lines, MCF-7 and T-47D. These data demonstrate that Notch3 induces GSK3 β expression. Notch3 overexpression downregulates the mesenchymal phenotype marker and upregulates the epithelial marker, which suggests that Notch3 may inhibit the progression of EMT in breast cancer.

Notch3 activates GSK3 β by directly binding to CSL-binding sites in the GSK3 β promoter

Based on our results that GSK3 β expression is regulated by Notch3 transcriptionally, we speculated on whether Notch3 bound the GSK3 β promoter. To search for a specific binding site in the GSK3 β promoter we found a sense binding site at position -1131 to -1126 and three antisense binding sites at -1165 to -1160, -308 to -303, and -167 to -162 (Fig. 3A). To investigate whether Notch3 binds to the endogenous GSK3 β promoter in chromosomal DNA, we performed a ChIP assay. We found that Notch3 interacts with the region between positions -368 to -268 containing a CSL antisense binding site (Fig. 3B).

To investigate the activity of the GSK3 β promoter directly regulated by Notch3, we constructed a reporter assay vector pGL3-GSK3 β -enhancer containing the region from -354 to -268 of the GSK3 β promoter. A dual-luciferase reporter assay was carried out to determine GSK3 β promoter activity in Notch3-overexpressing MDA-MB-231 cells, and Notch3-knockdown MCF-7 cells by co-transfection with a reporter assay vector and *Renilla* luciferase reporter genes. After Notch3 silencing in MCF-7 cells, the luciferase activity of the GSK3 β reporter decreased in a dose-dependent manner (Fig. 3C). Consistently, the luciferase activity of the GSK3 β reporter increased following N3ICD overexpression in MDA-MB-231 cells (Fig. 3D). Additionally, GSK3 β promoter activity showed no difference in a mutant CSL binding site reporter assay vector (Fig. 3C and D). These results indicated that Notch3 upregulated GSK3 β by directly binding to the GSK3 β promoter.

Ectopic Notch3 expression inhibits migration and invasion *in vitro*, which is attenuated by GSK3 β silencing

To investigate the effects of Notch3 overexpression or knockdown of Notch3 on migration and invasion by breast cancer cells, a wound healing assay, transwell *in vitro* migration, and invasion assays were performed. After cells were cultured for 48 h, the width of the wound in MCF-7 siRNA-Notch3 cells had reduced to only 25% compared to that at 0 h, while the width of the wound in siRNA-NC cells had reduced to about 54.5% compared to that at 0 h. This suggested that silencing of Notch3 disinhibited MCF-7 cells

that had migrated into the wound area. Conversely, the effect was reversed by ectopic GSK3 β expression as the width of the wound was restored to 43.5%, indicating that the increased migration caused by the silencing of Notch3 was mediated by GSK3 β (Fig. 4A and B). In contrast, we observed that after culturing for 24 h, overexpression of Notch3 inhibited MDA-MB-231 cell migration into the wound region compared with that of the control group (44% vs. 14.5%), while downregulation of GSK3 β expression reversed this (21.5%; Fig. 4E and F). We initially evaluated the effects of recombinant Notch3 and GSK3 β expression on breast cancer cell invasion using migration and invasion assays. Notch3 knockdown increased cell migration by 3.87-fold and 5.35-fold, respectively, which was partially eliminated by GSK3 β overexpression (Fig. 4C and D). In contrast, Notch3 overexpression reduced migration by nearly 80.9% and 78.43%, respectively, which was partially abrogated by GSK3 β silencing (Fig. 4G and H). These data demonstrated that Notch3 induces GSK3 β expression and inhibits wound healing and migration/invasion, an effect that can be attenuated by GSK3 β knockdown *in vitro*.

Positive correlation of Notch3 and GSK3 β expression in patients with breast cancer

We analyzed the protein levels of Notch3 and GSK3 β in the pathological parameters of 68 human breast cancer samples. Forty-three of the 68 specimens (64.8%) were found to be positive for Notch3 expression. Notch3 negative and positive specimens are shown in Fig. 5A and B, respectively. Positive GSK3 β expression was found in 40 of the 68 samples (58.8%). Glycogen synthase kinase-3-beta negative and positive samples are shown in Fig. 5C and D, respectively. Immunohistochemistry of Notch3 and GSK3 β in breast cancer specimens revealed that Notch3 and GSK3 β expression are positively associated ($r=0.416$, $P=0.001$; Table). In general, these data demonstrated that Notch3 correlated in a positive manner with elevated GSK3 β expression.

High expression of both Notch3 and GSK3 β mRNA predicts an improved prognosis in breast cancer patients

We first examined the prognostic effect of the expression of Notch3 and GSK3 β in www.kmplot.com. High expression of Notch3 ($P=4.1e-07$) and GSK3 β ($P=3.8e-05$) mRNAs correlated with an improved RFS for all patients with breast cancer (Fig. 6A, B). Restricting the analysis to intrinsic subtypes of breast cancer, we found that high expression of Notch3 correlated with a greater RFS for luminal A, luminal B, and human epidermal growth factor receptor 2 (Her2) subtypes but not the basal-like subtype compared to those with low expression of Notch3 (Fig. 6D, G, J, M). In addition, patients with luminal A, luminal B, and Her2 subtypes showed a superior RFS when GSK3 β was overexpressed but not the basal-like subtype (Fig. 6E, H, N, K). Notably, high mRNA expression of both Notch3 and GSK3 β predicted greater relapse-free survival in overall ($P=1.8e-05$) and luminal A breast cancer patients ($P=0.0082$; Fig. 6C, F), but not luminal B, basal-like or Her 2 subtypes (Fig. 6I, L, O). In conclusion, Notch3 and GSK3 β mRNA overexpression suggests a good prognosis for patients with breast cancer.

Discussion

The Notch receptor is a highly conserved type I transmembrane glycoprotein that is involved in cell differentiation, proliferation, and survival, which play important roles in various tumors [28]. Notch1–4 has a highly similar structure and corresponding functional role in mammals, but has a distinct function in normal breast development and breast cancer [29]. It has been identified that inhibition of Notch1 expression can reverse the EMT process of breast cancer cells, thereby inhibiting cell migration and invasion [30]. Furthermore, a clinical study found that Notch1 correlated to a poor prognosis in patients with breast cancer [31]. Notch2 overexpression predicts better overall survival in women with breast cancer, which is completely different from Notch1 [32]. A recent study showed the carcinogenic effect of Notch4. Zhou et al. revealed that Notch4 overexpression can promote the EMT of breast cancer cells and maintain the self-renewal properties of mesenchymal breast cancer stem cells [33], which is of concern and is associated with endocrine drug resistance in breast cancer.

Although the basic structure of Notch3 bears a resemblance to other Notch receptor members, many differences exist in the intracellular domain as well as a special shorter transcription activation domain. Several studies have indicated that Notch3 may play a considerable role in the formation of mammary epithelial cells during breast development [34]. Daniel et al. [35] revealed that Notch3 could label luminal progenitor cells and inhibit the proliferation of tumor cells. Cui et al. [13] found that Notch3 regulated cell senescence by regulating P21, thus inhibiting the occurrence and development of tumors. Similarly, Notch3 is associated with the inhibition of cell proliferation and apoptosis in HER2-negative breast cancer [36]. Our previous studies revealed that ER α inhibits EMT by suppressing Bmi1 [14], and Notch3 upregulates ER α [21]. Meanwhile, another study from our group showed that Notch3 can inhibit EMT in breast cancer epithelial cells by transactivating Kibra [15]. Therefore, Notch3 may have a pivotal role in tumor suppression, especially in breast cancer EMT.

The loss of E-cadherin expression is regarded as an essential event in EMT. It leads to disruption of epithelial cell polarity induced EMT [37] thus maintaining the mesenchymal phenotype and enhancing the invasion and metastasis of cancer cells [38]. A variety of molecular mechanisms can repress E-cadherin expression via transcriptional inhibition and promotor methylation. Doble et al. demonstrated that GSK3 β acts as an EMT suppressor of the zinc-finger transcriptional repressor, snail, which regulates epithelial marker E-cadherin [17]. These results indicated that both Notch3 and GSK3 β inhibit EMT in breast cancer and GSK3 β can physically bind and phosphorylate the intracellular domain of two Notch paralogs [18, 19]. However, the interaction of Notch3 and GSK3 β in EMT, a potential key step in breast cancer metastasis that contributes to breast cancer-related deaths, remains unclear.

Our results revealed that GSK3 β was highly expressed in the luminal-type breast cancer cell lines, MCF-7 and T-47D, but was expressed at low levels in the TNBC cell lines, MDA-MB-231 and BT-549. This is consistent with our previous findings that the expression pattern of Notch3 is similar to GSK3 β in these cell lines. In addition, our previous studies found that the CSL-binding element, GGGAA, participated in regulating the ER and GATA3 [21, 39]. In this study, we demonstrated that Notch3 bound to the GSK3 β promoter, which contains CSL-binding elements. Here, we found that the luciferase reporter activity of GSK3 β decreased in the form of a concentration gradient after Notch3 knockdown in MCF-7 cells. In

contrast, GSK3 β -mediated luciferase activity increased after N3ICD over-expression in MDA-MB-231 cells. Our study on its molecular mechanism revealed that inhibition of Notch3 downregulated GSK3 β mRNA and protein levels in MCF-7 cells significantly. Conversely, Notch3ICD overexpression upregulated that of GSK3 β in MDA-MB-231 cells as illustrated in Figure 7.

In summary, our research findings indicated that Notch3 represses the processes of EMT in breast cancer, which is consistent with our previous reports. Remarkably, this finding demonstrates for the first time that Notch3 may inhibit the EMT process in breast cancer cells through transcriptionally up-regulating GSK3 β . From data obtained from clinical tissue, Notch3 expression was significantly consistent with GSK3 β expression in breast cancer tissue samples. In a prognostic analysis, high expression of mRNA of both Notch3 and GSK3 β was related to better RFS in all patients with breast cancer studied, which implies that Notch3 and GSK3 β are beneficial biomarkers in this disease. Our results highlight a novel mechanism for exploring how Notch3 regulates EMT as well as the crosstalk between Notch and Wnt signaling pathways. This may have important implications for identifying new biomarkers for the prognosis of and as therapeutic targets in breast cancer.

Declarations

Funding

This work is partly supported by the funds from the Science and Technology Project of Xiamen Municipal Bureau of Science and Technology (Grant No. 3502Z20199047) and an open project of Xiamen's Key Laboratory of Precision Medicine for Endocrine-Related Cancers (Grant no. XKLEC 2020KF02).

Ethics approval

The work was conducted in accordance with the Declaration of Helsinki. The study was approved by the Medical Ethics Committee of the Cancer Hospital of Shantou University Medical College and Xiang'an Hospital of Xiamen University.

Competing interests

None of the authors have any financial or personal relationships with other people or organizations that inappropriately influenced this research.

Informed consent

This article does not contain any studies with human participants or animals. The entire study protocol was approved by the Medical Ethical Committee of the Cancer Hospital of Shantou University Medical College and Xiang'an Hospital of Xiamen University. The need for obtaining informed consent was waived by this committee.

Reference

References are not available with this version.

Table

Table. Association of Notch3 and GSK3 β expression in human breast cancer

Notch3	GSK3 β		χ^2	r	P
	+	-			
+	32	11	11.744	.416	.001
-	8	17			

Figures

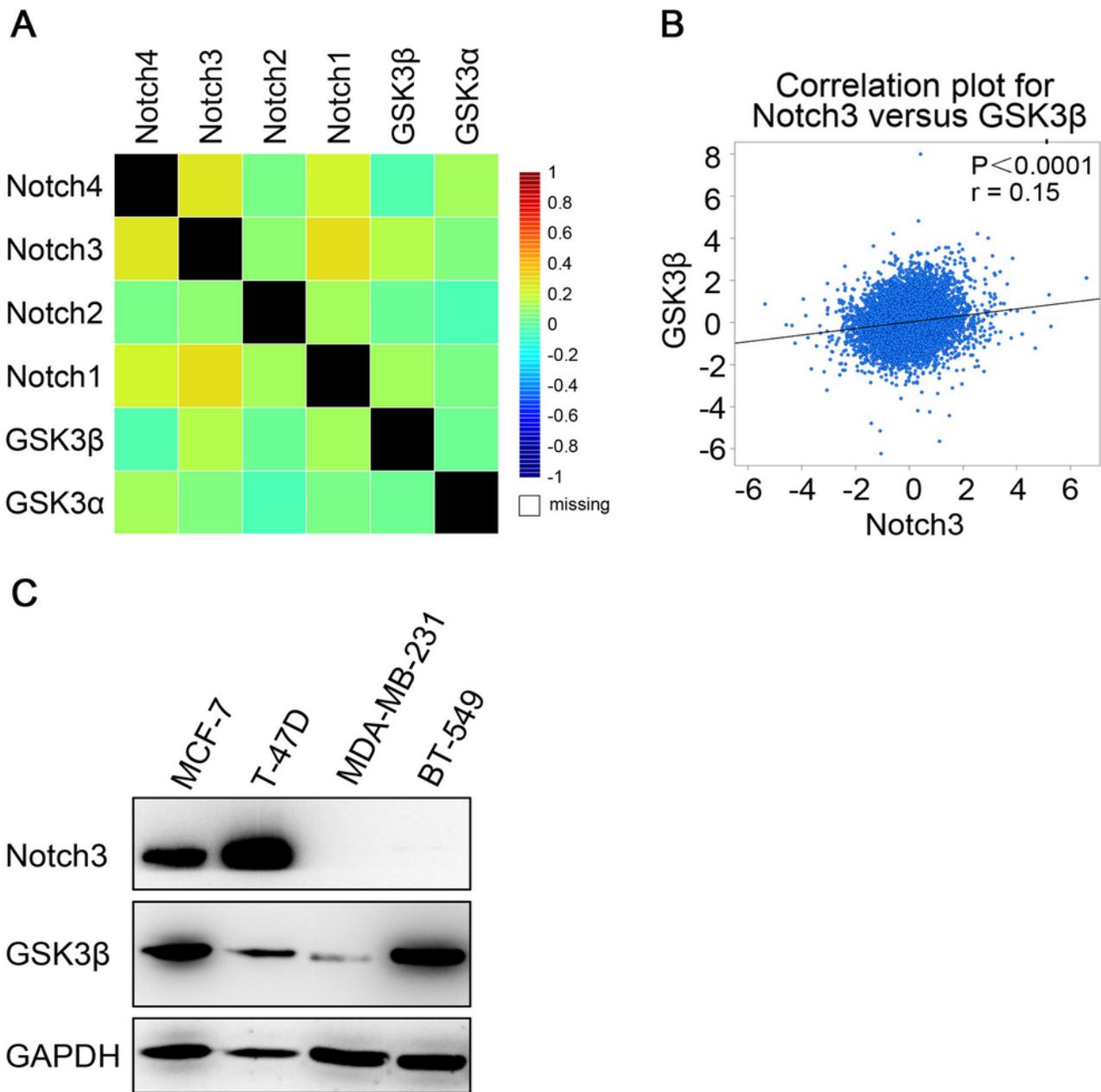


Figure 1

Figure 1

Notch3 is expressed in the luminal subtype and modulates GSK3β expression in breast cancer cell lines

A: Heat map representing the correlation of DNA microarrays of GSK3β and Notch1–4, which was obtained from Breast Cancer Gene-Expression Miner v4.7. **B:** A significant positive correlation existed

between Notch3 and GSK3 β . The correlation coefficient was $r=0.15$, $P < 0.0001$. **C:** Notch3 and GSK3 β expression in distinct subtypes of breast cancer cell lines detected by western blotting.

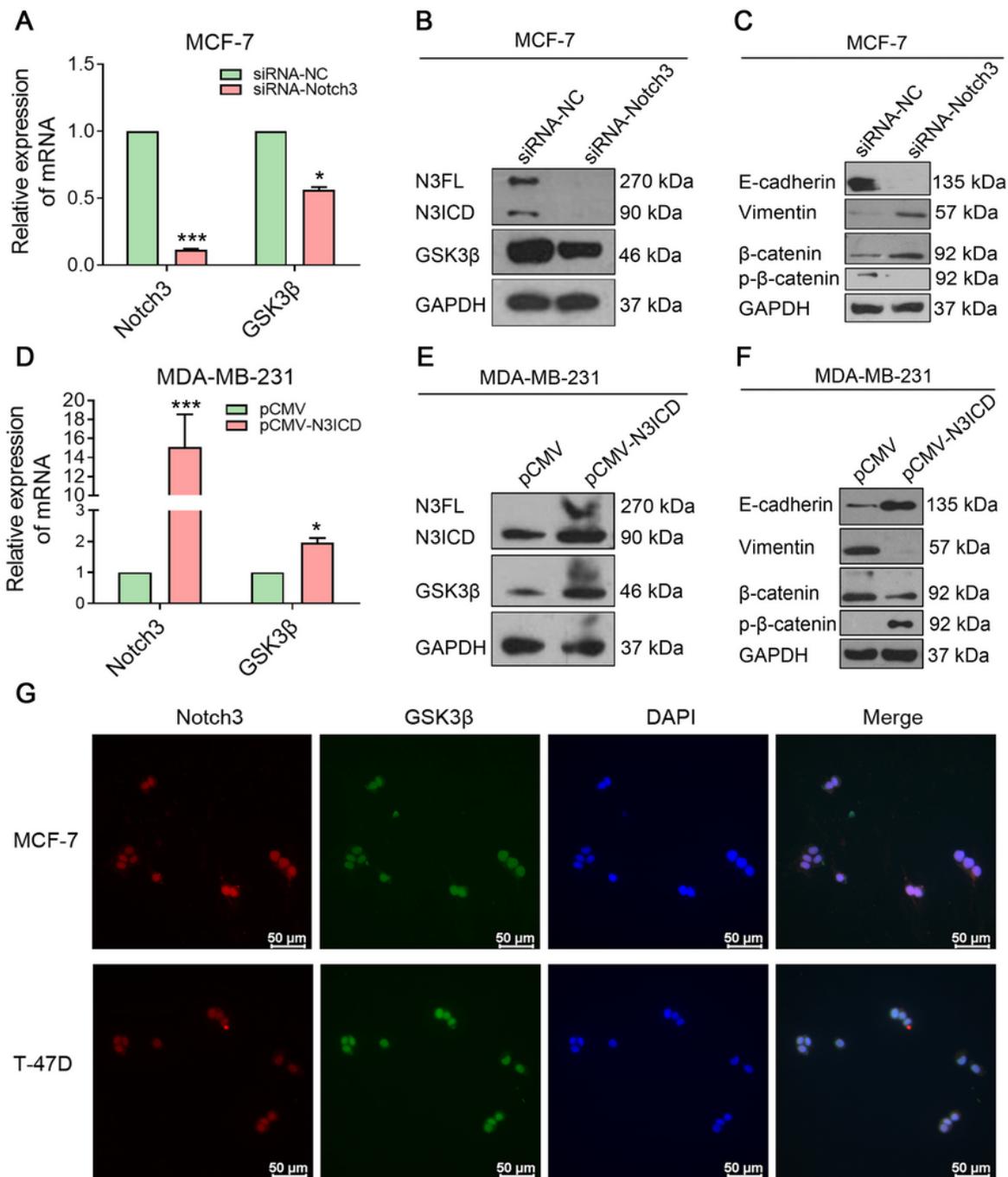


Figure 2

Figure 2

Ectopic Notch3 induces GSK3 β expression and inhibits epithelial–mesenchymal transition

A: MCF-7 cells were transiently transfected with small interfering (si) RNA of Notch3 or vehicle (control). After 48 h, quantitative reverse transcription (qRT)–PCR was used to assess the mRNA levels of Notch3 and glycogen synthase kinase-3-beta (GSK3b). (**B, C**): Protein levels of Notch3, E-cadherin, vimentin, β -catenin and phosphorylated (p)– β -catenin in MCF-7 cells transfected with siRNA-negative control (NC) or siRNA-Notch3 after 48 h were measured by western blotting. **D:** MDA-MB-231 cells were transiently transfected with PCLE-Notch 3 intracellular domain (N3ICD) or vehicle (control). After 48 h, qRT–PCR was used to assess the mRNA levels of Notch3 and GSK3b. (**E, F**): Protein levels of Notch3, E-cadherin, vimentin, β -catenin and p- β -catenin in MDA-MB-231 cells transfected with PCLE-N3ICD or vehicle (control) after 48 h were measured by western blotting. **G:** Immunofluorescence staining of GSK3 β and Notch3 in MCF-7 and T-47D cells. Nuclei were counterstained with DAPI. The scale bar represents 50 μ m. * $p < 0.05$, *** $p < 0.001$

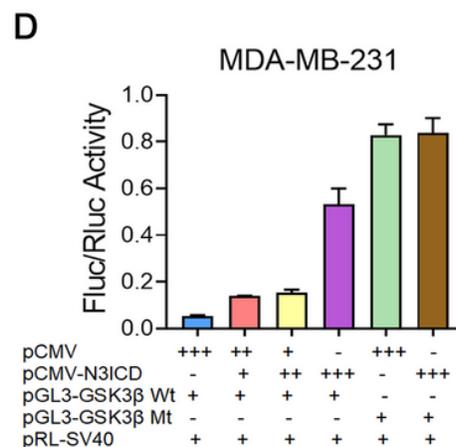
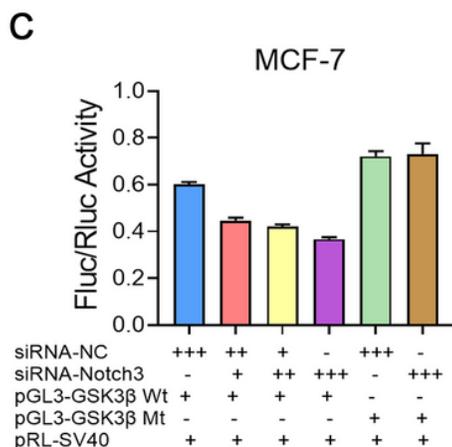
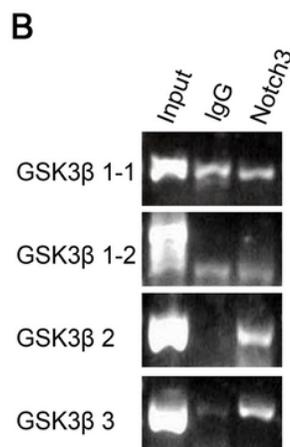
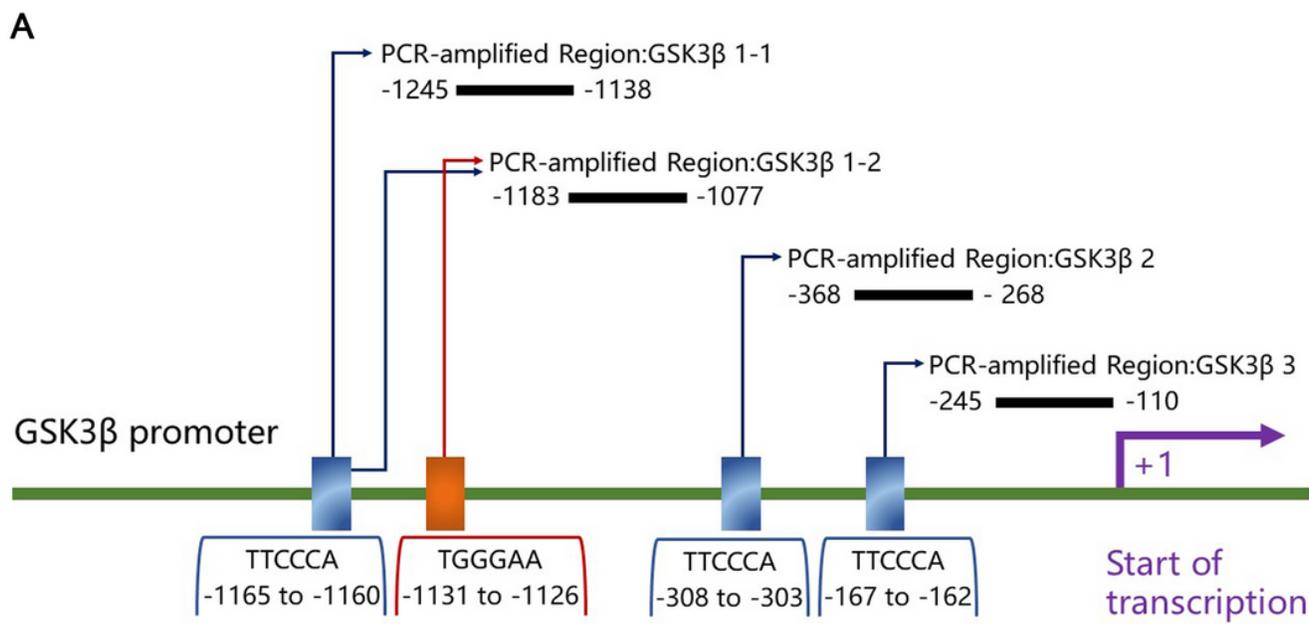


Figure 3

Figure 3

Notch3 transactivates GSK3β by directly binding to the GSK3β promoter

A: A schema of the four CSL-binding element-containing primers (glycogen synthase kinase-3-beta [GSK3β] 1-1, GSK3β 2, and GSK3β regions containing respective single CSL-binding elements; region GSK3β 1-2 containing two CSL-binding elements) used for chromatin immunoprecipitation (ChIP) assays. B: Products of ChIP were amplified by PCR and analyzed by 2% agarose gel electrophoresis. A specific band was seen in the GSK3β 2 region; C: MCF-7 cells co-transfected with gradient concentrations of siRNA-Notch3 ("+" 10 pmol, "++" 20 pmol, "+++" 40 pmol, "-" 0 pmol) or the same concentrations of

siRNA-negative control (NC) (“+” 20 pmol, “++” 30 pmol, “+++” 40 pmol, “-” 0 pmol), 10 ng pRL-SV40, and 200 ng wide-type pGL3-GSK3 β -enhancer (pGL3-GSK3 β -enhancer Wt) or 200 ng mutation-type pGL3-GSK3 β -enhancer (pGL3-GSK3 β -enhancer Mt). **D:** MDA-MB-231 cells co-transfected with gradient concentrations of pCMV- Notch3 intracellular domain (N3ICD; “+” 200 ng, “++” 400 ng, “+++” 800 ng, “-” 0 ng) or the same concentrations of pCMV (“+” 400 ng, “++” 600 ng, “+++” 800 ng, “-” 0 ng), 10 ng pRL-SV40, and 200 ng pGL3-GSK3 β -enhancer Wt or 200ng pGL3-GSK3 β -enhancer Mt. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

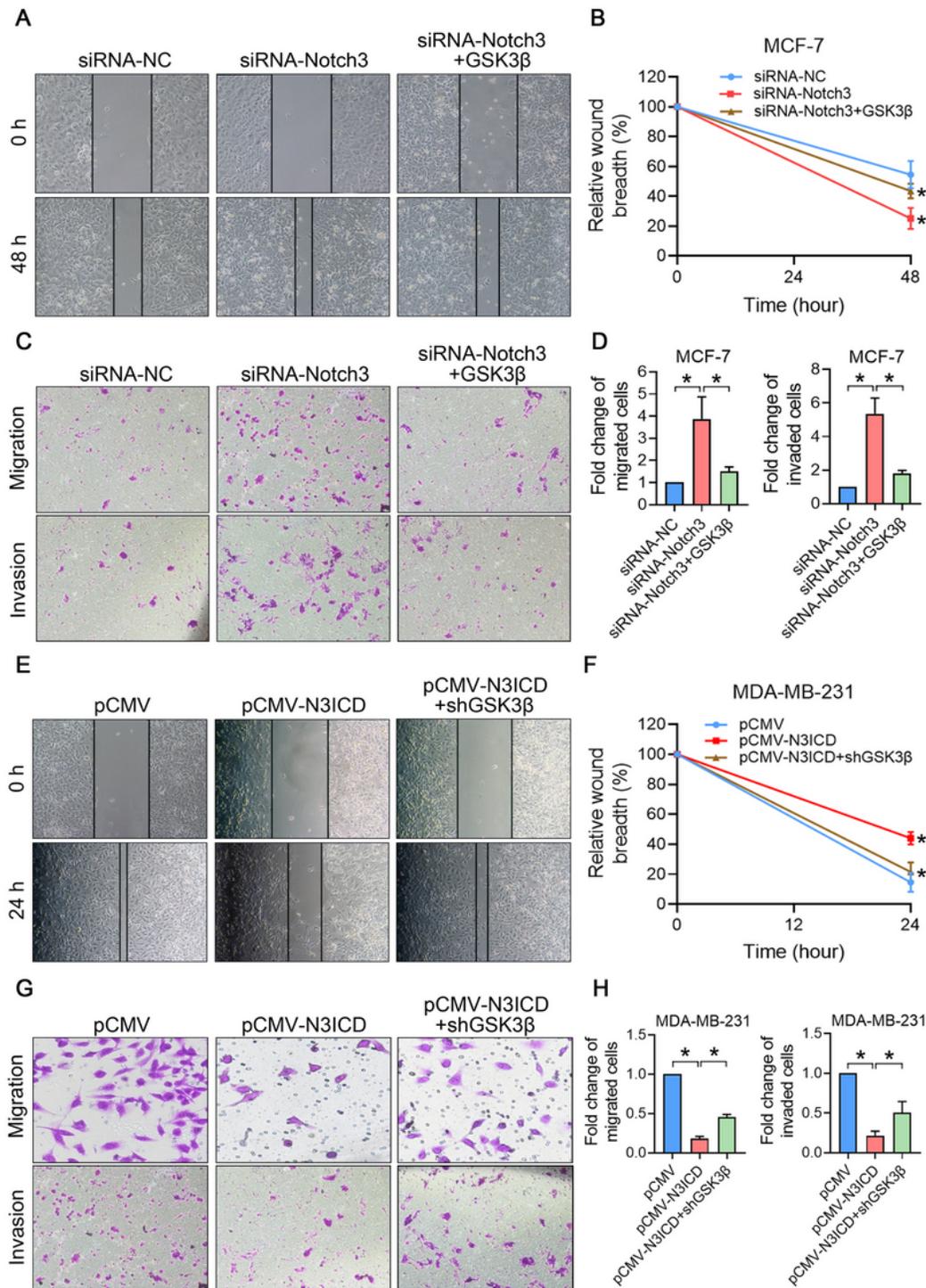


Figure 4

Figure 4

Restoration of GSK3 β expression reverses Notch3-mediated suppression of migration and invasion in breast cancer cells

A: Notch3 intracellular domain (N3ICD) knockdown induced MCF-7 cell growth *in vitro*. This effect was attenuated by glycogen synthase kinase-3-beta (GSK3 β) overexpression. **B:** Quantitative analysis of the wound healing rate of cells in each treatment group MCF-7siRNA-NC, MCF-7siRNA-Notch3 and MCF-7siRNA-Notch3+GSK3 β . **C, D:** MCF-7siRNA-Notch3, MCF-7siRNA-NC, and MCF-7siRNA-Notch3 +pCMV3-GSK3 β -GFP-Spark cells were subjected to transwell migration and invasion analysis. The mean \pm SD of migrated cells of three independent experiments is shown in the panel. **E:** N3ICD overexpression inhibited MDA-MB-231 cell growth *in vitro*. This effect was attenuated by GSK3 β knockdown. **F:** Quantitative analysis of the wound healing rate of cells in each treatment group above. **G, H:** MDA-MB-231pCMV, MDA-MB-231pCMV-N3ICD, and MDA-MB-231pCMV-N3ICD + psi-U6.1/eGFP/shRNA-GSK3 β cells were subjected to transwell migration and invasion analysis. The mean \pm SD of migrated cells of three independent experiments is shown in the panel. Magnification 400 \times . * $p < 0.05$. GFP, green fluorescent protein; NC, negative control; siRNA, small interfering RNA

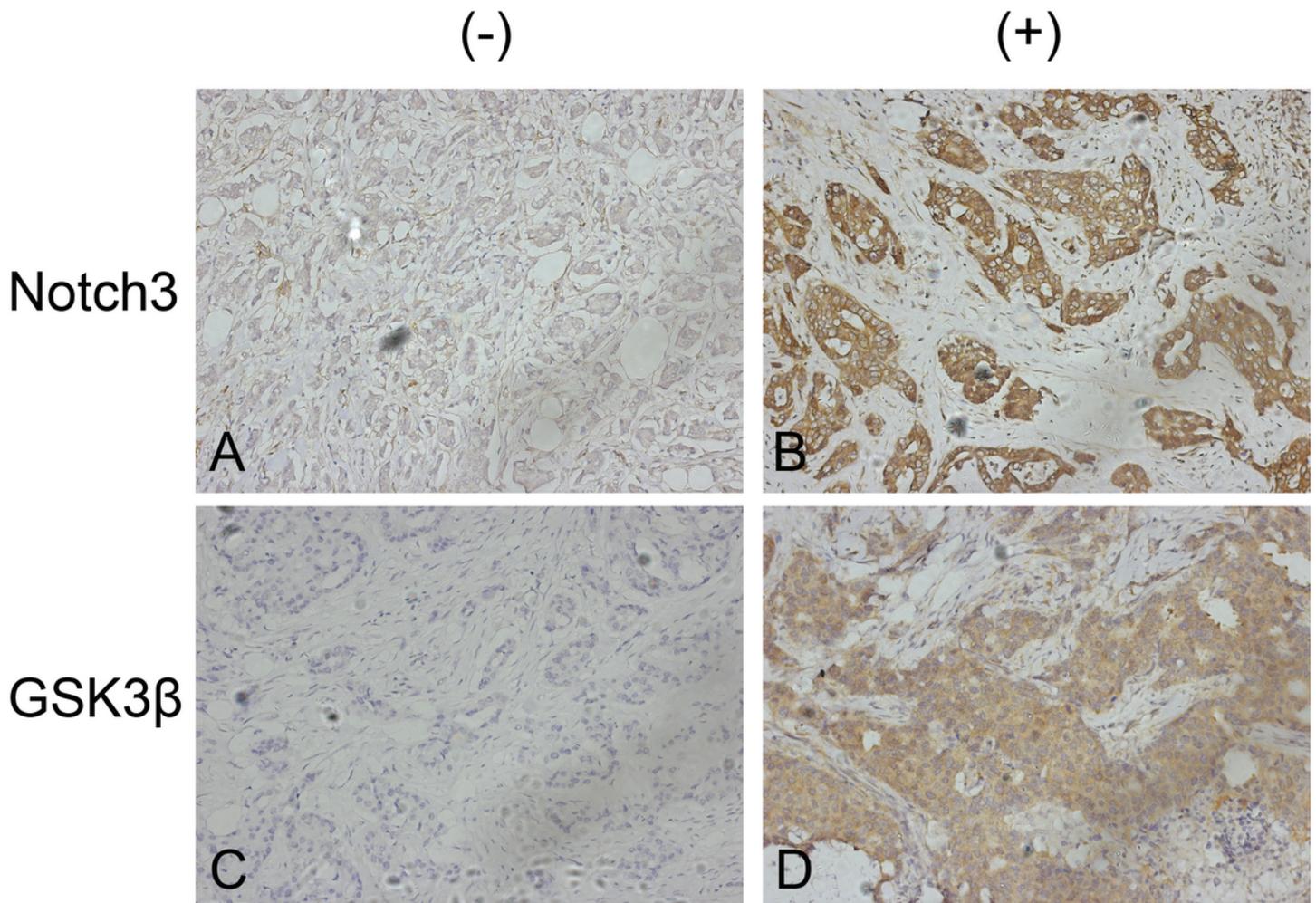


Figure 5

The correlation between Notch3 and GSK3 β expression in human breast cancer specimens

A: Representative image of Notch3 negative (-) staining cells in human breast cancer tissue. **B:** Representative image of Notch3 positive (+) staining cells. **C:** Representative image of glycogen synthase kinase-3-beta (GSK3 β) negative (-) staining cells. **D:** Representative image of GSK3 β positive (+) staining cells. Magnification 200 \times .

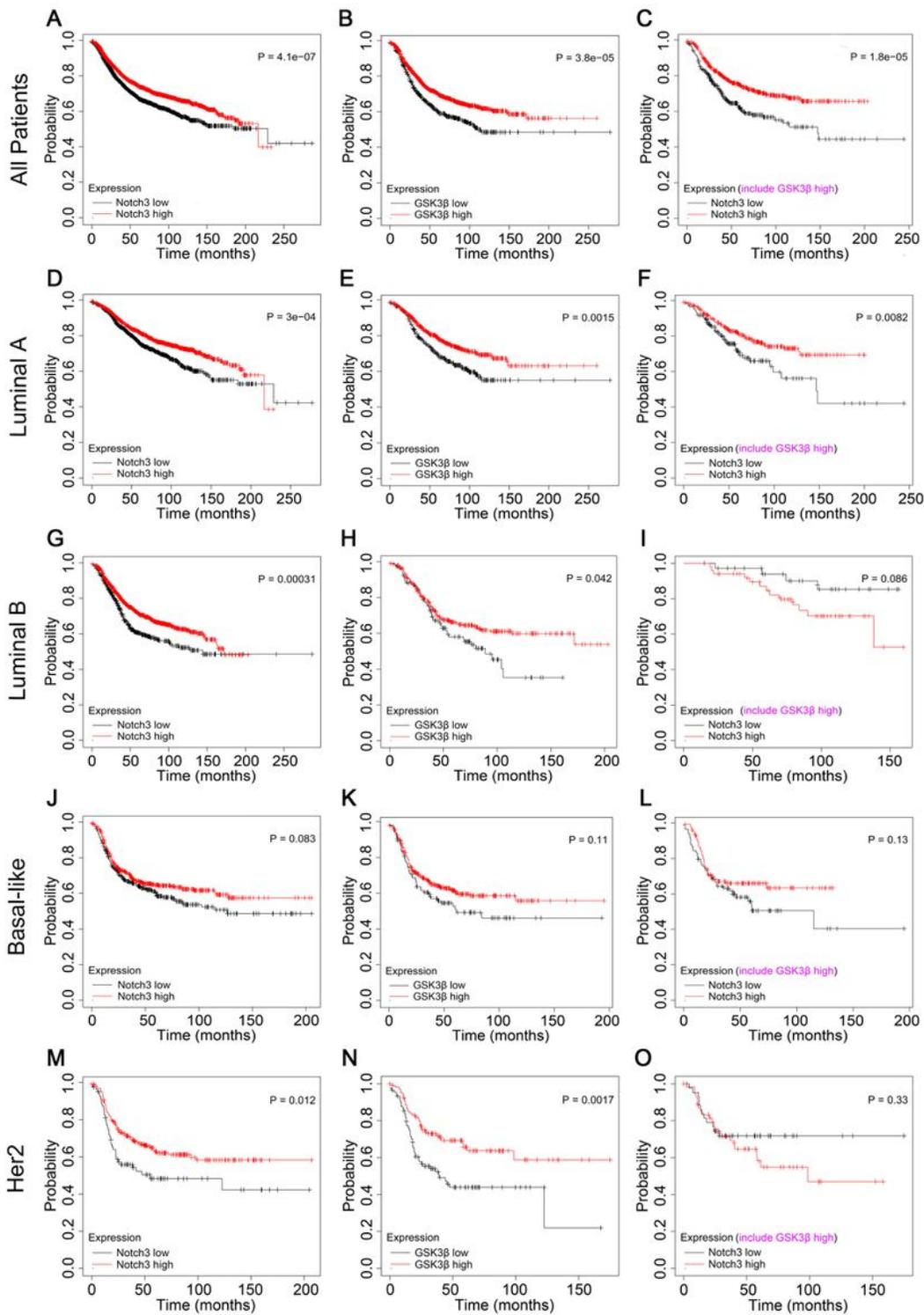


Figure 6

Figure 6

Analysis of Notch3 and GSK3β expression of RFS in patients with breast cancer

(A, D, G and M): High expression of Notch3 resulted in better recurrence-free survival (RFS) among different breast cancer subtypes but not the basal-like subtype (J). B, E, H, and N: High expression of glycogen synthase kinase-3-beta (GSK3β) resulted in a better RFS among different breast cancer

subtypes but not the basal-like subtype (K). A superior RFS was observed for breast cancer patients expressing both high Notch3 and GSK3 β levels in all patients, luminal A subtype (C, F) but not luminal B, basal-like, or Her 2 subtypes (I, L, and O).

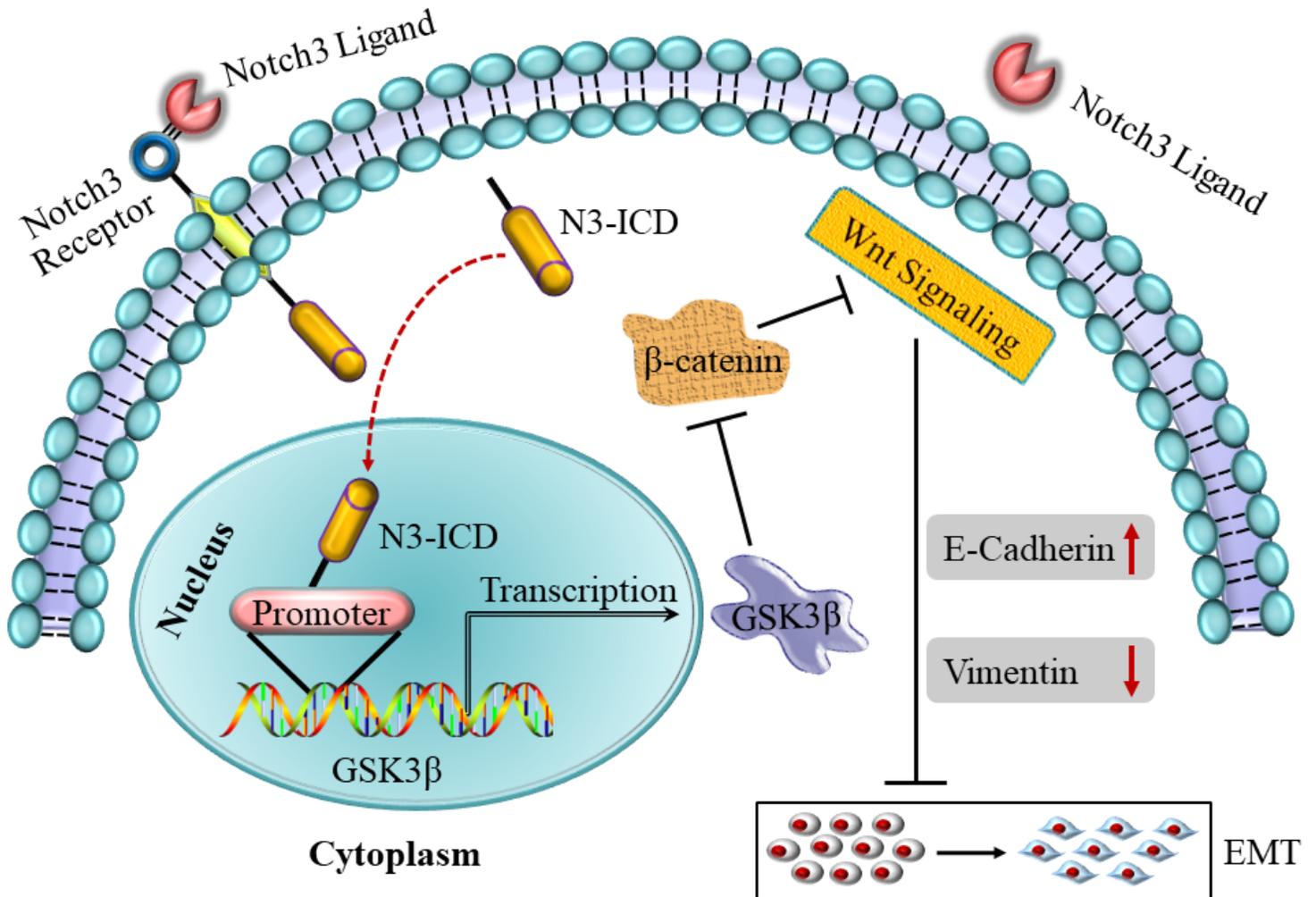


Figure 7

A Notch3ICD/GSK3 axis model that regulates EMT

Notch3 receptor binds ligand and Notch3 intracellular domain (Notch3ICD) is excised. Notch3ICD binds to CSL-binding elements in the glycogen synthase kinase-3-beta (GSK3 β) promoter, transactivates GSK3 β and its downstream pathway, and inhibits epithelial-to-mesenchymal transition (EMT) in breast cancer.

Supplementary Files

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